ENHANCEMENT OF BIOCELLULOSE PRODUCTION IN MIXED MEDIUM CULTURE

SIEW KAH WENG

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UNIVERITI MALAYSIA PAHANG

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ABSTRACT

In this research, the results for biocellulose production by *Acetobacter xylinus* in mixed culture medium were reported. Biocellulose production was determined by utilizing different feedstocks of single sugars and sugar mixtures which were applied according to certain glucose to fructose ratio. Data for pH changes and the biocellulose production from every medium culture were thoroughly analyzed. In this experiment, it was ensured that all the samples had the initial temperature and pH of 30ºC and pH 5.5 respectively. The temperature was kept constant throughout the whole experiment while the changed pH value was taken as final pH at the end of the experiment for every sample. The highest production using sugar mixtures of 1:9 glucose to fructose ratio was 1.57g/L. The final pH values recorded in the different sugar mixtures were in the range of 4.0–5.5. The lowest final pH of 4.56 was determined in the medium that contained a single carbon source of glucose, as most of the glucose was converted into gluconic acid and lead to lowest biocellulose production of 0.69g/l. In contrast, the highest pH value of 5.3 was determined in the medium that contained a single carbon source of fructose and lead to the higher biocellulose production of 0.9g/l. Analyzing profiles for final pH and biocellulose production for the medium with higher glucose concentration showed that the glucose was preferable to be converted to gluconic acid rather than biocellulose synthesize. Besides, it was also determined that biocellulose production rate in mixed culture medium was higher than in culture medium that only consist of a single carbon source and this had proved that the experiment of enhancing biocellulose production with mixed medium culture was applicable. Results reported in this study demonstrated that the production of biocellulose can be enhanced by using carbon sources mixture with a suitable ratio. This not only represented that biocellulose would be a renewable source of cellulose in the future, but also might lead to major improvements in production if proper supplements and control were utilized in the fermentation process.

ABSTRAK

Kajian ini memberikan laporan tentang keputusan penghasilan biosellulosa yang dihasilkan oleh *A. xylinum* di dalam medium kultur campuran. Biosellulosa yang dihasilkan dengan menggunakan gula tunggal dan gula campuran mengikut nisbah glukosa kepada fruktosa yang telah ditetapkan sebagai bahan mentah di dalam kultur media. Data untuk perubahan nilai pH dan penghasilan biosellulosa dalam setiap kultur media dianalisis. Di dalam eksperimen ini, suhu dan pH medium kultur dipastikan dalam 30°C dan pH 5.5. Selepas itu, suhu kultur media dipastikan malar sepanjang eksperimen dijalankan. Untuk pH pula, nilai pH yang telah berubah untuk setiap kultur media telah dicatatkan pada akhir eksperimen. Penghasilan biosellulosa yang paling tinggi didapati daripada kultur media yang mengandungi campuran gula dalam nisbah 1:9 glukosa kepada fruktosa iaitu sebanyak 1.57 g/L. Semua nilai pH untuk pada akhir eksperimen untuk setiap sampel ialah dalam lingkungan pH 4-5.5. Walau bagaimanapun, nilai pH yang terendah didapati pada akhir eksperimen ialah pH 4.6 yang didapati dari kultur media yang hanya mengandungi satu sumber karbon glukosa sahaja. Selepas itu, kultur media tersebut menghasilkan jumlah biosellulosa yang terendah iaitu sebanyak 0.69 g/L. Sebaliknya, nilai pH yang paling tinggi didapati dari kultur media yang hanya mengandungi satu sumber karbon iaitu fruktosa sahaja. Kultur media ini menghasilkan jumlah biosellulosa yang lebih banyak iaitu sebanyak 0.9 g/L. Dengan mangkaji dan memperbandingkan semua semua hasil biosellulosa daripada setiap kultur media, adalah didapati bahawa penghasilan asid gluconic lebih diutamakan daripada penghasilan biosellulosa apabila kandungan glukosa dalam media kultur semakin bertambah. Tambahan lagi, ia juga didapati bahawa kadar penghasilan biosellulosa di dalam kultur media campuran yang mengandungi campuran glukosa dan fruktosa sebagai sumber carbon adalah lebih tinggi daripada kultur media yang hanya mengandungi satu jenis sumber karbon. Ini telah membuktikan bahawa usaha untuk meningkatkan penghasilan biosellulosa dengan menggunakan kultur media campuran adalah berjaya. Keputusan eksperimen menunjukkan penghasilan biosellulosa boleh ditingkatkan dengan menggunakan kultur media campuran dan nisbah campuran sumber carbon yang sesuai. Ini bukan hanya menunjukkan biosellulosa sebagai sumber sellulosa yang boleh diperbaharui pada masa hadapan, tetapi juga menunjukkan bahawa jumlah penghasilan biosellulosa boleh ditingkatkan lagi dengan menggunakan bahan mentah dan pengawalan yang sesuai dalam proses penapaian.

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LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
×	Times
Wt%	Weight Percentage
β	Beta

LIST OF ABBREVIATIONS

A.	*Acetobacter*
BC	Biocellulose
CO2	Carbon Dioxide
DA	Dalton
FTIR	Fourier Transformed Infrared Spectrocopy
IR	Infrared
M	Molarity
O2	Oxygen
SEM	Scanning Electron Microscopy
WAC	Water Absorption Capacity
Wh	Hydrated Weight
Wd	Dried Weight
3-D	Three Dimensional

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

In this chapter, an overview of the study will be introduced. The title of my study is the 'Enhancement of Biocellulose Production in Mixed Medium Culture'.

1.2 BACKGROUND OF STUDY

Anselme Payen was the French Chemist who found out the existence of cellulose in 1838. Samples are taken from the plant matters and the chemical formula of cellulose was designed (Klemm *et al.*, 2005). Thermoplastic polymer (celluloid) was the first cellulose production by Hyatt Manufacturing Company in 1870 (Raymond, 1986). The polymer structure of cellulose was determined by Herman Staudinger in 1920. It was first chemically synthesized in 1992 by Kobayashi and Shoda (Klemm *et al.*, 2005).

Biocellulose is a form of cellulose product, produced by a specified bacteria. It is also called microbial cellulose. It was first recognized as cellulose in 1886 (Kuga and Brown, 1988). The bacteria which can produce cellulose are from the genera *Aerobacter, Acetobacter, Achromobacter, Agrobacterium, Alacaligenes, Azotobacter, Pseudomonas, Rhizobium* and *Sarcina* synthesize cellulose (Ross *et al.*, 1991). However, Acetobacter is the only species which can synthesis enough cellulose for commercial purposes. *Acetobacter xylinum* is the mostly used bacteria species for commercial interest. *A. xylinum* is recently renamed as *Glucoacetobacter xylinus* (Ross, 1991).

In a survey, it shows that the industry produces 200000 tons of biocellulose in 2006 and is targeted to be reaching 5000000 tons during 2015 align with the increasing world demand for biocellulose products as is estimated that the world market will face the problem of inadequate fossil feedstock in the next 10 to 20 years (Williams, 2006). Hence the aim of this research is to enhance and improve the production of biocellulose align with the rapid increasing demand of biocellulose products.

1.3 PROBLEM STATEMENT

Most of the industrial polymer productions such as plastic products are giving negative impacts towards our mother nature. Many of the synthetic polymers nowadays are manufactured from petrochemicals and are non-biodegradable (Gautam, 2009). Plastics are a product of polyethylene polymer (from fossil resources) that is unable to be biodegraded. The continuous usage of non-biodegradable products will lead to environmental pollutions. A research done in 2009 has determined that about eight billion plastic bags are used in Malaysia (Rajeswary and Himanshu, 2010). Even there is recycling system, there is not much plastic is being recycled and pollutions still going on, contributes to greenhouse effect. There are over 380 billion plastic bags are used annually but only 5.2% is sent for recycling, so the others plastics are left on earth forthousands years (Thangham, 2007).

Moreover, fossil carbon source is limited and will out of stock one day (Steinbüchel, 2005). Hence, cellulose which is biodegradable is the next polymer that will replace the non-biodegradable polymer. However, cellulose is gained form plants and will damage the Mother Nature too if trees are cut off to obtain cellulose. Therefore, biodegradable biocellulose that is produced by bacteria (*A. xylinum*) is preferred to reduce the consumption of trees. In the future, degradable polymers will be replacing the today's commercialized plastic products in market (Gautam, 2009). Therefore, ways for enhancement of biocellulose production are significant as the bio cellulose is the alternative polymer which will be used worldwide in the future and using mixed medium culture is one of the biocellulose enhancement efforts.

1.4 RESEARCH OBJECTIVE

The main objective of this study on Enhancement of Biocellulose in Mixed Medium Culture is to determine for the most suitable carbon sources mixture composition to enhance the production of biocellulose in mixed medium culture.

1.5 SCOPE OF STUDY

This research is based on experimental studies of biocellulose production, using mixed medium culture. To achieve the objectives mentioned above, three scopes have been identified:

i. To produce biocellulose using fructose and glucose which are used to prepare the mixed medium culture.

ii. To analyze the properties of biocellulose using FTIR.

iii. To characterize the morphology of the produced biocellulose by using Scanning Electron Microscope.

1.6 RATIONALE AND SIGNIFICANCE OF STUDY

Normally, single sugar is used in preparing the medium for biocellulose production. For example, biocellulose is produced from medium culture containing glucose with *Acetobacter xylinum* (Masaoka *et al.*, 1992)**.** In this research, the medium culture is prepared using fructose glucose mixture in different ratio. The ratio of the two components in the mixture that will produce the highest amount of biocellulose is determined in the end of the research and will be recommended for the usage in the real polymer industry. It will be a new era for polymer industry.

In the previous research which was done by Yaser Dahman, Kithsiri E. Jayasuriya and Magdalina Kalis, the biocellulose production rate in mixed medium culture was higher than the biocellulose production rate in single culture medium because the analyzed data proving that big sum of the metabolized sugars are mostly synthesized for bacterial cell growth and maintenance but not for biocellulose

production in medium culture with single sugars, causing low cellulose production. On the other hand, sugar was consumed was for synthesizing biocellulose production with sugar mixtures (Dahman *et al.*, 2010). This is very helpful information for the effort to enhance the biocellulose production in this era. Hence in order to be more advance in the effort of biocellulose production, this research has been done by using the mixed culture medium too but this research is a further on project as the optimum ratio of two carbon sources in the mixed culture medium which will produce the maximum amount of biocellulose is the main target to be determined.

As biocellulose is the alternative polymer in the future, it has a high potential to be commercialize in a big scale. Hence, the enhancement of biocellulose production is for the increasing demand for biocellulose products (Joong, 2001). Biocellulose can be used to produce biodegradable plastic products, facial mask, biopaper and and even used in medical field (Rainer and Farah, 1998**).** The market of bioprocess product is developing rapidly.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

A review of literature is performed to identify studies relevant to the topic. The main source for the literature search is the Science Direct website. The review is organized chronologically to offer insight to how past research efforts have laid the groundwork for subsequent studies, including the present research effort. The review is detailed so that the present research effort can be properly tailored to add to the present body of literature as well as to justly the scope and direction of the present research effort.

2.2 BIOCELLULOSE

Cellulose is a polymer that we can be determined in most of plants. On the other hand, biocellulose is the cellulose produced by bacteria; Acetobacter species called *Acetobacter xylinum* or recently is called *Gluconacetobacter xylinus* (Ross *et al.*, 1991). Similar to cellulose, biocellulose is a biodegradable polymer which is used to produce environment friendly products. Biocellulose is mainly designed to replace the usage of cellulose to produce things such as paper and also to replace the usage of non-biodegradable polyethylene polymer for plastic products. This is because the production of papers needs a lot of trees to be cut off and this is proved to be harmful to environment since long time ago (Sangok and Shoda, 2005). Moreover, the usage of non-biodegradable polymer products can be on Earth for thousands of years as pollutants (Gerald, 2008). Therefore, an end must be put on this after the usage of biocellulose products are introduced worldwide and this will absolutely reduce the

pollution rates. This is significant as the pollution on our earth nowadays has reached its critical level.

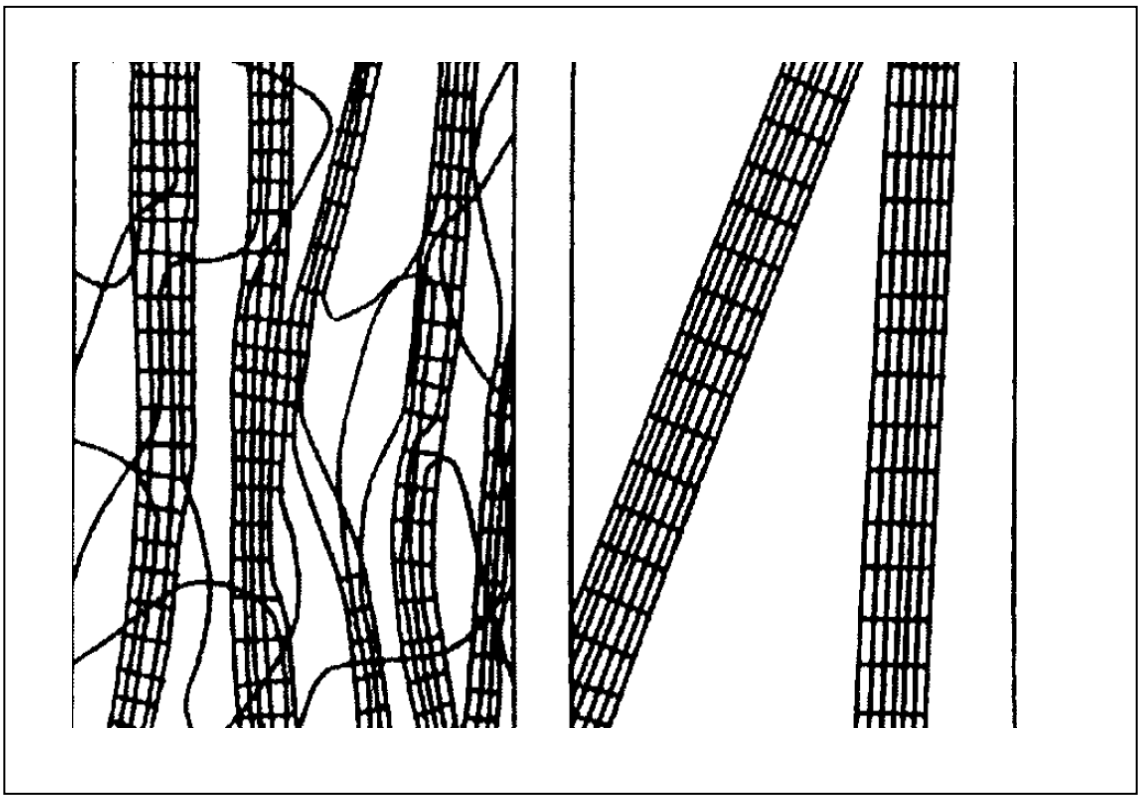

Figure 2.1:Schematic model of biocellulose micro fibrils (right) drawn in comparison with the 'fringed micelles' of plant cellulose (left) fibrils

Source: Iguchi *et al.* (2000.)

(a)

(b)

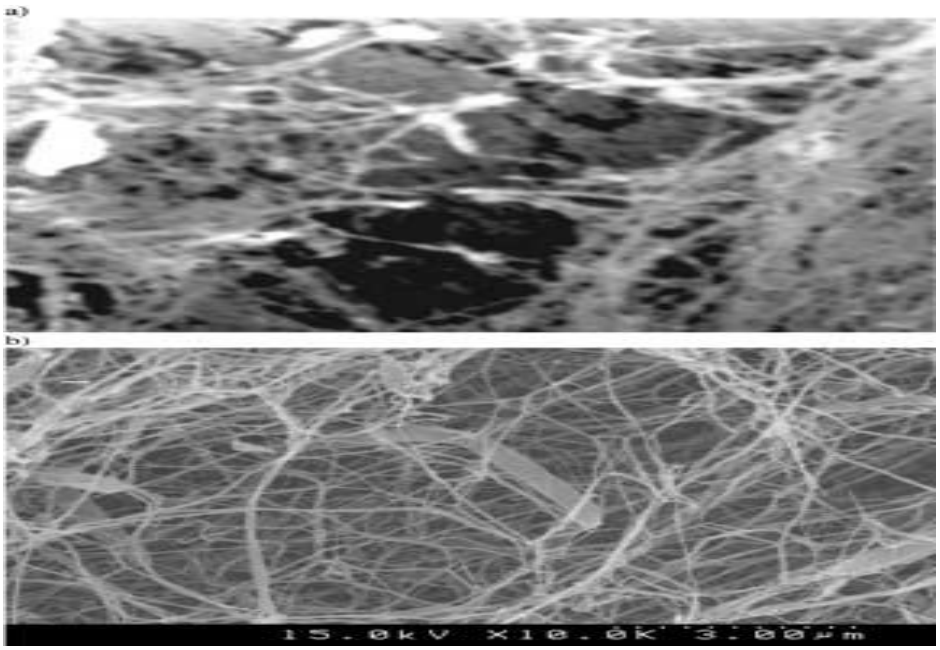

Figure 2.2: Scanning Electron Microscopy images of BC membrane from static culture of *A. xylinum* (a) and bacterial cells with attached cellulose ribbons (b)

Source: Stanislaw *et al.* (2010)

From Figure 2.1, we can observe the structure differences between biocellulose and Plant Cellulose. It shows that the plant cellulose is associated with naturally formed lignin and hemicellulose and they are hard to be removed. This means that plant cellulose is harder to be purified than biocellulose. Then from Figure 2.2, we can notice that the Biocellulose is in the form of fibrils on the surface of the medium culture via the microscopic image. The second microscopic image shows that the rod-shaped Acetobacter *xylinum* is within the biocellulose fibrils.

The biocellulose structure begins to be formed in its actual biosynthesis when the carbon compounds within the culture medium are utilized by *A. xylinum*. After that, it is polymerized and becomes single, linear b-1,4-glucan chains and is excreted into the surrounding medium via a linear row of pores, situated on the outer membrane. The assemble of the b-1,4-glucan chains in the outer part of the cell is a precise, hierarchical process. Then, they start to form subfibrils that consist of 10–15 nascent b-1,4-glucan

chains, microfibrils, and lastly becoming bundles of microfibrils, containing loosely wound ribbon which is consisting of an estimated amount of 1000 individual glucan chains (Ross *et al.*, 1991). Consequently, a thick, gelatinous membrane is formed in the static culture conditions as we can see in Figure 2.3. It is characterized by a 3-D structure comprising of an ultrafine network of cellulose nanofibres (3–8 nm) that are very uniaxially oriented (Czaja *et al.*, 2004).

Sucha 3-D structure which cannot be found in plant cellulose can bring about high cellulose crystallinity (60–80%) and powerful mechanical strength for biocellulose. Biocellulose is stronger than plant cellulose but the biocellulose fibrils are about 100 times smaller than that of plant cellulose. Hence, this unique nano-morphology gives rise to a bigger surface area which enables biocellulose to keep a larger amount of water (up to 200 times of its dry mass). In addition to that, biocellulose also performs great elasticity, high wet strength, and conformability. The small size biocellulose fibrils is determined to be the main factor that bring about its incredible compound for wound healing system. Moreover, the unique property of the biocellulose makes it to be a never-dried cellulose membrane which is a very nano-porous substance. For example, biocellulose enable antibiotics to be transferred in to the wound and be the protecting barrier against any external infection (Bielecki *et al.*, 2002). Different from plant cellulose, biocellulose is thoroughly free of lignin and hemicelluloses which can be observed in Figure 2.1 above.

2.2.1 Chemical Structure

Considering the chemical structure of cellulose, it is a homopolymer comprising of glucose glycosidically attached in a b-1_4 conformation while the repeating unit of the polymer synthesis comprises of two glucose molecules linked together under 180 degrees rotation with each other. Biocellulose has a similar chemical structure to that of plant cellulose but differs from their degree of polymerization (Jonas and Farah, 1998). A long straight unbranched polymer chain is produced by the bonding among glucose units in cellulose and the capacity to produce intermolecular hydrogen bonds between adjacent glucan chains is very high. Ribbons of microfibrils are synthesized at the surface of *A. xylinum* cellulose. The dimensions of the ribbons are 3–4 nm thick and 70–80 nm wide and the hydrophobic bonds are used to maintain the shape of the microbial

cellulose sheet (Chawla *et al.*, 2008). Experiment has been brought out and it is concluded the occurring of the initial development of inter- and intramolecular hydrogen bonds in cellulose sheet lead to the formation of cellulose crystalline structure (Bielecki *et al.*, 2005). The existence of tunnels as observed by scanning electron microscope (SEM) argues for some kind of coordination during the pellicle formation and a random formation of cellulose microfibrils (Shibazaki *et al.*, 1995).

X-ray diffraction can be used to distinguish two common crystalline forms of cellulose designated as I and II (Kuga *et al.*, 1993). The microbial cellulose observed under SEM showed a significant difference in appearance of the external and internal surfaces of the pellicles. The external surfaces had irregular clusters of fibrils, whereas internal surfaces were organized in fractured sections. The relative reactivity of the OH groups in the glucose residues has been determined to decrease in the order of 6'OH>2'OH>3'OH. Furthermore, the nitration rate is highly depends on the concentration of nitric acid in the process. The 6'OH groups in the crystalline and disordered components are subjected to nitration at nearly the same rate during lower concentrations (Chawla *et al.*, 2008). These two components are randomly spread in the entire partition of each microfibril. On the other hand at higher concentration, all OH groups go through nitration very fast. There is no regioselective reactivity being identified among the three kinds of OH groups in solid-phase acetylation and this may because of the characteristic reaction which precedes in a very thin layer between the acetylated and nonacetylated regions in each microfibril (Yamamoto *et al.*, 2006)).

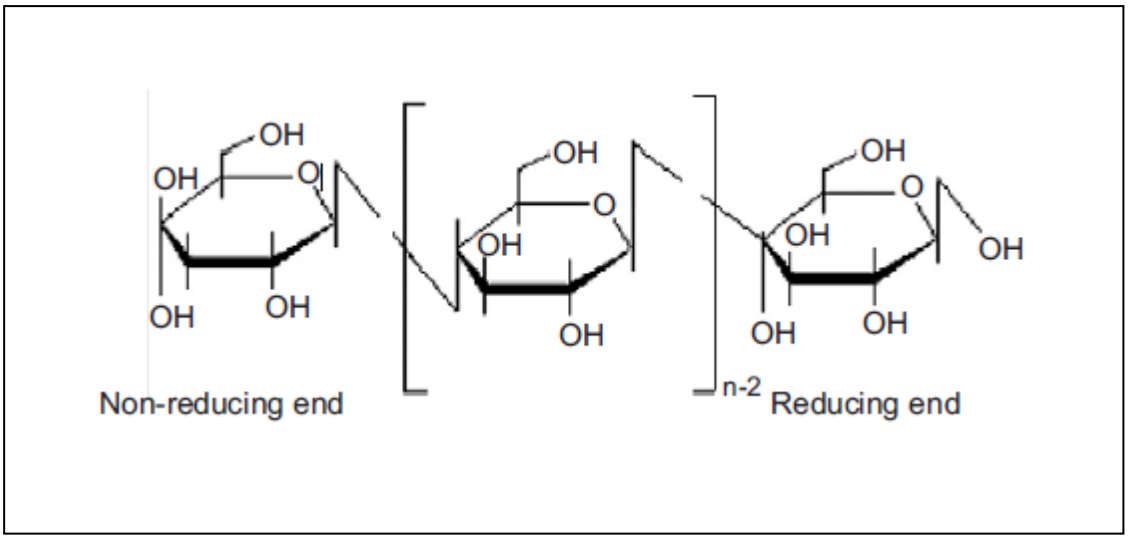

Figure 2.3: Repeating units of cellulose

Chawla *et al.* (2008)

2.2.2 Biocellulose Properties

Biocellulose has high tensile strength, crystallinity, moldability and high degree of polymerization as well as insolubility in most of the solvents (George *et al.*, 2005). Normally, biocellulose fibrils are 0.1–10 mm thick which is a hundred times thinner than that of plant cellulose fibrils with good shape retention. Moreover, its water adsorption capacity is over 100 times higher by mass and it is also much stronger than plant cellulose (Schrecker *et al.*, 2005). Macroscopic morphology of cellulose strictly depends on the culture conditions, which can easily be tailored for the physicochemical properties. It has been reported that cellulose fibre has molecular weight of approximately 142.73 kDa and possesses the degree of polymerization of 793 (Wanichapichart *et al.*, 2002). It can be dissolved in concentrated acids such as nitric, sulphuric or hydrochloric acid as well as soluble in 8.5 % NaOH solution. The solubility of cellulose in the alkali can be increased by adding 1 % of urea to the solution (Laskiewicz, 1998)). Despite of the higher stability of alkali-treated cellulose membrane which can withstand temperature in the range of between 343 and 370 °C, it can be degraded at a higher temperature above 300 °C. Composites prepared by adding bacterial cellulose and microfibrillated cellulose (MFC) processed through fibrillation of kraft pulp were compared for mechanical properties and it is determined that the

bending strength increased up to 425 Mpa, while the Young's modulus increased from 19 to 28 Gpa, nearly retaining the modulus of the bacterial cellulose sheets (Nakagaito *et al*., 2005).

The uniform nano-scalar network structure is the special characteristic of the biocellulose that lead to its mechanical properties which oriented bi-dimensionally when compressed. The swelling property of cellulose under different conditions has been studied and it has been observed that. NaOH at lower concentration brings about greater swelling in fibres comparing with other alkalis at the same concentrations (George *et al*., 2005). From the observation, it is determined that the percentage mass gain by the cellulose membranes after soaking in different alkaline solution is following the order of NaOH>KOH>Na2CO3>K2CO3. The pervaporation characteristics of deproteinated microbial cellulose membrane are investigated over a wide range of water-ethanol feed composition and it has been found to be promising for dehydration of azeotropes of ethanol and it has a high selectivity towards water at a reasonable flux (Dubey *et al.,* 2002). The cellulose membrane as a molecular separation medium is its basic characteristic in aqueous conditions and it is well defined with the modification with chemical treatments to control its molecular permeation characteristics (Shibazaki *et al.,* 1999). Biocellulose possesses an interesting character that is the ability to control and modify not only the physical characteristics but also the chemical composition of the cellulose fibre (Shirai *et al.,* 1994). Direct dyes such as amide black and trpan red, fluorescent brightening agents or derivatives like carboxymethyl cellulose can be applied to alter the structure of the cellulose assembly (Cousins *et al.,* 1997). *A. xylinum* which is cultivated in Hestrin-Schramm (HS) medium that comprises of acetyl glucomannan avoids the assembly of cellulose microfibrils and the crystal structure of cellulose is changed (Shakairi *et al.,* 1998). In addition to that, loose bundles of cellulose microfibrils also can be observed when *A. xylinum* is cultivated in Hestrin-Schramm medium which consists of glucuronoxylan because glucuronoxylan in the medium prevents the assembly of cellulose microfibrils and changes the crystal structure of cellulose too (Chawla *et al.,* 2008*).* In contrast, pectin which presents in the HS medium helps in assembly of cellulose fibrils but no obvious effect is observed (Tokoh *et al.,* 2002).

2.3 BACTERIA THAT SYNTHESIZE CELLULOSE

Table 2.1: Biocellulose producers

Genus	Cellulose structure
Acetobacter	extracellular pellicle composed of ribbons
Achromobacter	fibrils
Aerobacter	fibrils
Agrobacterium	short fibrils
Alcaligenes	fibrils
Pseudomonas	no distinct fibrils
Rhizobium	short fibrils
Sarcina	amorphous cellulose
Zoogloea	not well defined

Source: Jonas and Farah (1998)

Table 2.1 above shows a variety of bacteria which can synthesize cellulose. The table also indicates that the produced cellulose structure is different for each type of bacteria.

2.3.1 *Acetobacter xylinum*

Acetobacter xylinum is the most suitable bacteria used for the production of commercialized biocellulose. It is also known as *Acetobacter xylinum* or *Gluconacetobacter xylinus. Acetobacter* bacteria are normally found to have symbiotic relationships with various plants like sugarcane and coffee plants (Muthukuramasamy *et al., 2002*). *Acetobacter xylinum* is a gram-negative, aerobic bacterium which has been used as a model organism for the study of bacterial cellulose synthesis since long time ago; mainly because *Acetobacter xylinum* is able to produce a large quantity of biocellulose compare with other bacteria (Mayer *et al.*, 1991). For instance, a single *A. xylinum* cell is able to polymerize 200 000 glucose molecules per second into β-1,4 glucan chains that are then secreted to the surrounding medium, forming biocellulose in bundles-like shape (Ross *et al.*, 1991). The biocellulose fibres are synthesized in the membrane by cellulose synthase and are excreted through a row of 50 – 80 pore-like

synthetic sites (Delmerl and Amor, 1995). The formation of this floating cellulose matrix is thought to allow *A. xylinum*, an obligate aerobe, to grow in the higher oxygen tension at the surface of the medium. The cellulose synthase operon (*asc*) has been characterized, showing that the operon contains three genes, acsAB which codes for a 168 kDa polypeptide which is the cellulose synthase and acsC as well as acsD which are involved in cellulose production and crystallization (Saxena *et al*, 1994). The figure 2.4 below obviously shows that the *Acetobacter xynilum* is a rod-shaped bacteria. The biocellulose fibres also can be noticed in the surrounding of the bacteria in the medium.

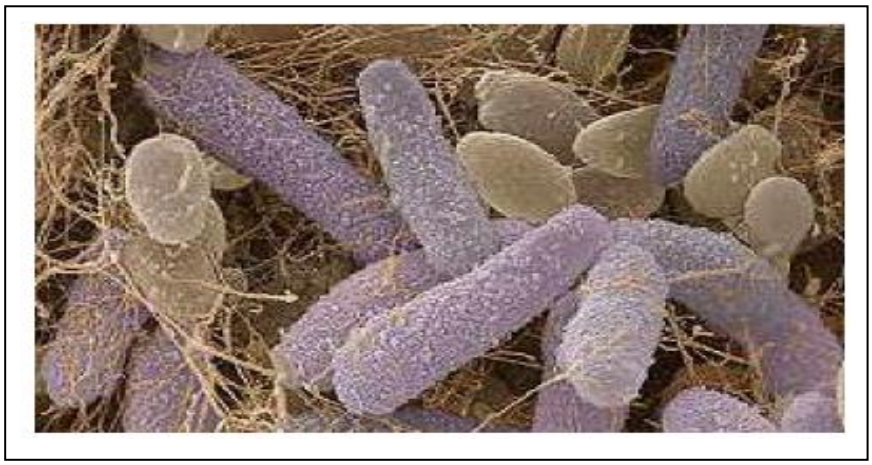

Figure 2.4: *Acetobacter xylinum* within the biocellulose fibre

Source: Norhayati (2009)